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Research article

Physico-chemical and ultra-structural characterizations of PLGA-loaded nanoparticles of Boldine and their efficacy in ameliorating cisplatin induced hepatotoxicity in normal liver cells *in vitro*

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Abstract

Production of unwanted hepatotoxicity and side-effects often restrict use of cisplatin as a chemotherapeutic agent in oncology. Therefore, this study was conducted for testing if co-administration of poly (lactic-co-glycolide) (PLGA)-loaded Boldine (NBol), an active alkaloid ingredient of Boldo (*Peumus boldus)* plant reported to have cytoprotective property could alleviate cisplatin-induced toxicity better than Boldine (Bol) in normal liver cells (WRL-68) but not protecting liver cancer cells (HepG2). NBol were prepared by adopting the standard solvent displacement method and characterized for their size, morphology and zeta potential by using dynamic light scattering, atomic force-, scanning- and transmission-electron microscopies, and fourier transform infra-red spectroscopy. Cellular entry was also demonstrated with the aid of fluorescence microscopy. The entrapment efficiency, percent yield, *in vitro* release kinetics and protective effects of NBol were analyzed by studying cellular morphology of liver cells, MTT assay, and cytotoxicity assay. Expression of different proteins related to cytotoxicity and cell survival were critically studied by western blotting and confocal microscopy. Boldine loaded PLGA nanoparticles were successfully prepared with 84.19 ± 1.72% yield in the nanometer range and had 81.93 ± 0.915% encapsulation efficiency. Co-administration of NBol and cisplatin produced better and faster site-specific action than that of Bol due to former's smaller size (115.5±0.469nm) and negative zeta potential (-17.4±2.38 mV). Co-administration of NBol protected normal cells from cisplatin-induced hepatotoxicity, but had no or negligible protective effects in cancer cells. Thus, NBol had greater potential of being used as a supportive therapy along with cisplatin in the treatment of cancer.

Key words: PLGA- Nano-encapsulation, Boldine, Cisplatin, Hepatotoxicity, DNA-targeting; Cytoprotection.

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1. Introduction

Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 [1]. Surgery, chemotherapy and radiotherapy, are not always capable of addressing the problem of such a magnitude, and newer avenues must be explored utilizing knowledge emerging in other medical regimens such as complementary and alternative medicines (CAMs), at least for use as supportive medicines for alleviating toxicity produced by conventional therapy [2]. Till now, chemotherapy is considered as one of the best treatment methods to combat cancer. However, cytotoxicity generated by most orthodox chemotherapeutic drugs often restricts their use considerably. Cisplatin is one such drug that has a strong anti-cancer effect, but its use often becomes limited for its severe toxic side-effects. It is preferentially taken up and accumulated in the liver and kidney cells, causing suppression of synthesis of different anti-oxidant enzymes [3], and thereby resulting in generation of reactive oxygen species (ROS)-related toxicity [4] and cell death [5]. In recent years, new brand of relatively less toxic or non-toxic natural agents, mainly from the plant resources are being harnessed, which have less cytotoxic effects on normal cells, but have strong anti-cancer effects.

A low dose of Boldo (*Peumus boldus*) extract has recently been reported by us [2] to significantly reduce cisplatin toxicity in normal cells, increasing their survivability, but did not quite affect cisplatin's cytotoxicity in cancer cells. In furtherance of this work, we have now examined if Boldine (chemically Boldine ([s]-2,9-dihydroxy- 1,10-dimethoxyapor phine) (Bol, Figure 1A), a major bioactive alkaloid separated from Boldo crude extract reported to have anti-oxidant and radical-scavenging properties [6], can show similar or stronger anticancer effect at a still lower dose. Therefore, we first evaluated cytotoxicity of Boldine, if any, on liver cancer cells (HepG2) and in normal liver cells (WRL68) *in vitro* and then its ability, if any, to reduce cisplatin's cytotoxicity generated in normal liver cells without affecting cisplatin's cytotoxic effects in HepG2 cancer cells. On obtaining satisfactory results, our next goal was set to formulate nano-encapsulation of Boldine (NBol) by a suitable biodegradable polymer, poly (lactic-co-glycolide) (PLGA), and to examine if it could provide greater bioavailability by faster cellular uptake. First, we characterized the nanoparticles physically and microscopically, and evaluated relative cytoprotective potential of Boldine (Bol) and nano-Boldine (NBol) against cisplatin-induced cytotoxicity in normal liver cells.

Thus, the main objectives of the present study were to i) examine, if Boldine had cytoprotective effects in normal liver cells against cisplatin-induced cytotoxicity/ hepatotoxicity; ii) to attempt effective nano-encapsulation with PLGA, a non-toxic biodegradable polymer and to characterize the PLGA-loaded nanoparticles physico-chemically by utilizing dynamic light scattering (DLS) for determination of particle-size and zeta potential of the nanoparticles, and iii) to determine the entrapment efficiency, percent yield, time for cellular entry, and

in vitro release kinetics; and iv) to evaluate the efficiency of nano-Boldine in target-specific drug delivery, its relative efficacy in drug release and ability to protect normal liver cells preferentially against cisplayin-induced cyto- and hepato-toxicity, and finally, v) to analyze the possible signaling pathway(s) involved in achieving this goal.

2. Materials and Methods

Chemicals and Reagents

Pure form of Cisplatin [cis-Diamineplatinum(II) dichloride] and Boldine [$C_{19}H_{21}NO_4$[p-code 1001359485] were purchased from Sigma (USA). Dulbecco's modified Eagle medium (DMEM) and the antibiotics, namely, penicillin, streptomycin, and neomycin (PNS) were purchased from HiMedia, India. Fetal bovine serum (FBS), trypsin and ethylene di-amine tetra-acetic acid (EDTA) were procured from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were bought locally from Tarson, India. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were obtained from Sigma, USA. Fluorescein isothiocyanate (FITC) and primary antibodies were obtained from Santacruz Biotechnology Inc, USA. Secondary antibodies were purchased from Sigma, USA. All organic solvents used were of HPLC/analytical grade.

Preparation of blank nanoparticles and Boldine loaded nanoparticles

Boldine loaded nanoparticles were prepared by following the solvent displacement method [7]. At first, 10 mg Boldine was dissolved in 3 ml acetone and then 50 mg PLGA was further added to dissolve in the mixture. This organic phase mixture was added in a drop-wise manner (0.5 ml/min) to 20 ml of aqueous solution containing the stabilizer, F68 (1% polyoxyethylene–polyoxypropylene; w/v). The mixture was then stirred at 400 rpm by a laboratory magnetic stirrer at room temperature till the organic solvent was completely evaporated. The stabilizer was then removed from the nanoparticles by centrifugation at 25,000×g at 4 ◦C for 30 min. After that the pellet was re-suspended in Mili-Q water and washed thrice. Blank nanoparticles were also prepared in the same manner, but without adding Boldine.

Atomic force microscopic (AFM) studies:

Surface morphology and particle size of the nanoparticles were determined using atomic force microscopy. Samples for AFM imaging were prepared by placing a drop of NBol suspension on a freshly cleaned mica sheet and letting it dry up in the air. Observation was recorded through AFM (Veeco di CP-11) imaging in amplitude, tapping and deflection modes.

Particle size determination by dynamic light scattering (DLS) method:

The mean particle size and distribution, as well as the zeta potential of the Boldine-loaded PLGA-nanoparticles were determined by a dynamic light scattering technique using a Zetasizer Nano-ZS (Malvern Instruments, South borough, UK). The intensity of scattered light was detected at 90° to an incident beam. The freeze-dried powder was dispersed in aqueous solution and measurements were done, after the aqueous micellar solution was filtered with membrane extruder having an average pore size of 0.22 ml (Millipore, St. Charles, MO). Data were analyzed in the automatic mode. Size was determined from the average value of 20

runs, with triplicate measurements within each run. Zeta potential of the blank nanoparticles was also determined in the same way.

Determination of NBol loading and encapsulation efficiency

2 mg of NBol was dissolved in 2 ml acetone to extract Boldine into acetone for the loading and encapsulation estimations. The samples in acetone were quietly shaken on a shaker for 12 h at room temperature and 48 h at 4°C. These solutions were centrifuged at 1000g and supernatant was collected. The suspension (10 µl) was diluted to 1 ml with acetone and was used for the estimations. Boldine concentrations were determined by a UV/VIS spectrophoto meter (SHIMADZU UV- 1700) at 269 nm and 337 nm characteristic absorption peaks of Boldine [8]. The Boldine loading content and encapsulation efficiency (EE) were calculated according to the following formula:

EE (%) = [(drug fed = drug loss) / (drug fed)]×100.

In vitro release kinetics

Boldine loaded PLGA nanoparticles were evaluated for their in vitro release kinetics of Boldine. Since Boldine is poorly soluble in water, Boldine released in aqueous buffer can easily be quantified after its separation. 50 mg of PLGA nanoparticles encapsulating Boldine was dispersed in 10 ml of PBS, pH 7.4. The solution was divided into 20 micro centrifuge tubes (500 µl each). The tubes were kept in a thermo stable water bath set at room temperature. The samples were kept in an orbital shaker maintained at 37°C ± 0.5°C stirring at 100 g. This was centrifuged at 5000g for 10 min to separate the released (pelleted) Boldine from the loaded nanoparticles. The released Boldine was re-dissolved in 1 ml of ethanol and the absorbance was measured spectrophoto metrically at 450 nm. The concentration of the released Boldine was then calculated using standard curve of Boldine in ethanol. The percentage of Boldine released was determined from the following equation:

Release (%) = [Boldine] released/ [Boldine] total×100

where, [Boldine] released is the concentration of released Boldine collected at time t and [Boldine] total is the total amount of Boldine entrapped in the nanoparticles.

Determination of percent yield

The dried nanoparticles were weighed and respective yields were calculated by using the following equation:

%Yield = [(weight of nanoparticles obtained)/(weight of drug and polymer used for nanoparticle-preparation)] × 100.

Scanning electron microscopy

The morphological characteristics of NBol were examined by field emission scanning electron microscope (FESEM) at an accelerating voltage of 20 kV. The samples were prepared by drop-casting NBol solutions on a cover slip which was subsequently dried for overnight in a desiccator. The completely dried sample was coated with gold.

Transmission electron microscopy

The samples were prepared by drop-casting dilute solutions of NBol on carbon coated copper grids and then dried overnight in desiccators. A Hitachi TEM operated at an accelerating voltage of 200 kV was used for taking the images.

Cellular uptake of Bol and NBol by fluorescence microscopy

The cellular entry of Bol and NBol in WRL-68 cells was analyzed by fluorescence microscopy [9]. The cells were treated with Bol and NBol (27μg/mL) for different time periods. The incubated cells were washed twice with PBS and examined under fluorescence microscope (Leica, Germany).

Cell culture and cytotoxicity determination

Cancer cells from the human hepato-cellular carcinoma cell line (HepG2) and normal liver cell line (WRL-68) were collected from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic (PNS) in a humidified incubator with 5% CO2. Cells were harvested with 0.025% trypsin-EDTA in phosphate buffer saline (PBS) and required number of cells allowed to adhere.

HepG2 and WRL-68 cells were treated separately with different concentrations of cisplatin (5–25 μmol/L). We determined the 50% lethal dose (LD50) (20 μmol/L) of cisplatin in these two cell lines, and because the LD50 for both cell lines was the same, we selected it for all other experiments. Then we treated HepG2 and WRL-68 cells with different concentrations of Bol (0-90μg/mL) and NBol (0-90μg/mL) in combination with LD50 dose of cisplatin(20 μmol/L) to examine if these could reduce the cisplatin-induced toxicity in these two cell lines. HepG2 and WRL- 68 were treated with only Bol (0-142μg/mL) or NBol (0-142μg/mL) to test if they had their own cytotoxic effect. Medium containing PLGA blank nanoparticles suspended in PBS served as control. Cytotoxicity assays were conducted in 96-well flat bottom microtiter plates with a cell density of 1×103 cells per well. After drug treatment, plates were incubated for 24 h. Thiazolyl blue tetrazolium bromide (MTT) was then added to each well at a concentration of 10 μmol/L and the plates were incubated in the dark for 2–3 h at 37 °C. To stop the reaction and develop a blue colour, acidic isopropanol was used. The absorbance value was recorded at 595 nm in a microtiter plate reader [10].

Experimental design and doses

Experimental sets were divided into 4 different groups: control (PBS treated), cisplatin treated (20μmol/L), cisplatin (20μmol/L) plus Bol treated (27μg/mL) and cisplatin (20μmol/L) plus NBol treated (27μg/mL).

Cellular morphology study

For study of morphology of cells, HepG2 and WRL-68 cells were placed in 6-welled plates and incubated with drug doses described above. After incubation for 24 h, cells were digitally photographed with a phase-contrast microscope (Leica, Germany), and morphological changes observed were recorded and photographed.

Determination of inter-cellular GSH

Reduced glutathione (GSH) content of WRL-68 cells was determined using the method of Hissin and Hilf (1976) [11]. According to this protocol, 2×105 cells per well were added to 6-well plates. Each well was added with control, or cisplatin, or Bol, or NBol, or cisplatin plus Bol, or cisplatin plus NBol, respectively and incubated for 24 h. The concentration of inter-cellular GSH was determined relative to a GSH standard and expressed

as percentage values with respect to the control treatment.

Immunoblot analysis

Proteins were extracted from cells of different experimental sets to check the activity of bax and cytochrome c (mitochondrial and cytosolic), caspase 3 (cytosolic), glyceraldehyde 3-phosphate dehydrogenase (GAPDH). 50 µg of protein lysate was denatured using Laemmli buffer and subjected to SDS-PAGE (sodium dodecyl sulfate poly-acrylamide gel electrophoresis) on a 12% agarose gel. The separated proteins were transferred to a polyvinyl difluoride membrane followed by blocking with 3% bovine serum albumin (BSA; w/v) in TBST (Tris-buffered saline and Tween 20) for 1 h. The membrane was probed with primary antibody (Santacruz Biotechnology, USA) overnight at 4 ºC followed by 3 h incubation with alkaline phosphatase (ALKP)-conjugated anti-mouse IgG secondary antibodies (Sigma, USA). Colour was then developed using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) in tris-buffered saline (TBS) solution.

Cytochrome c release study by confocal microscopy

The normal cells (WRL-68) were prepared for the immunofluorescence analysis using MitoTracker Red (50 nm) and secondary fluorescent FITC anti-goat antibody (Santa Cruz Biotechnology, USA). For confirmation of localization of cytochrome c in mitochondria and cytosol, control, cisplatin, cisplatin with Bol and cisplatin with NBol treated cells were incubated for 24 h with primary antibody (Santa Cruz Biotechnology, USA) at 4^{o} C overnight and developed with secondary FITC conjugated anti-cytochrome-c antibody. Then photographs were taken under confocal microscope (Carl Zeiss LSM 510 META Laser Scanning Microscope).

Statistical analysis

All data are expressed as mean±standard errors of three sets of data. Statistical significance was evaluated with analysis of variance (ANOVA) using SPSS Version 20. Comparisons among individual treatment groups were made by comparing data by the least square division (LSD) method. For all statistical tests, the threshold of $P<0.05$ was used to determine significance.

3. Results

Characterization of NBol

Boldine loaded PLGA nanoparticles were successfully prepared with 84.19 ± 1.72% yield in the nanometer range and had 81.93 ± 0.915% encapsulation efficiency. Images related to physical characterization of the nanoparticles including its chemical structure have been furnished in Figures 1A-D and Figures 2A-D. The mean diameter of NBol was determined to be 115.5 ± 0.469 nm with 0.21± 0.015 ploydispersity index (PDI) and -17.4 ± 2.38 mV zeta potential. AFM study displayed a spherical shape of nanoparticles with smooth surface without any pinholes or cracks (Figure 1B). The 2D Fast Fourier Transformation (FFT) image (Figure 1C) and line measuring method (Figure 1D) in AFM also showed uniform spatial frequency of topographic planar signal of NC. According to results of TEM study NBol had spherical shape, without cracks and core size- 15.2 nm (Figure 2D).

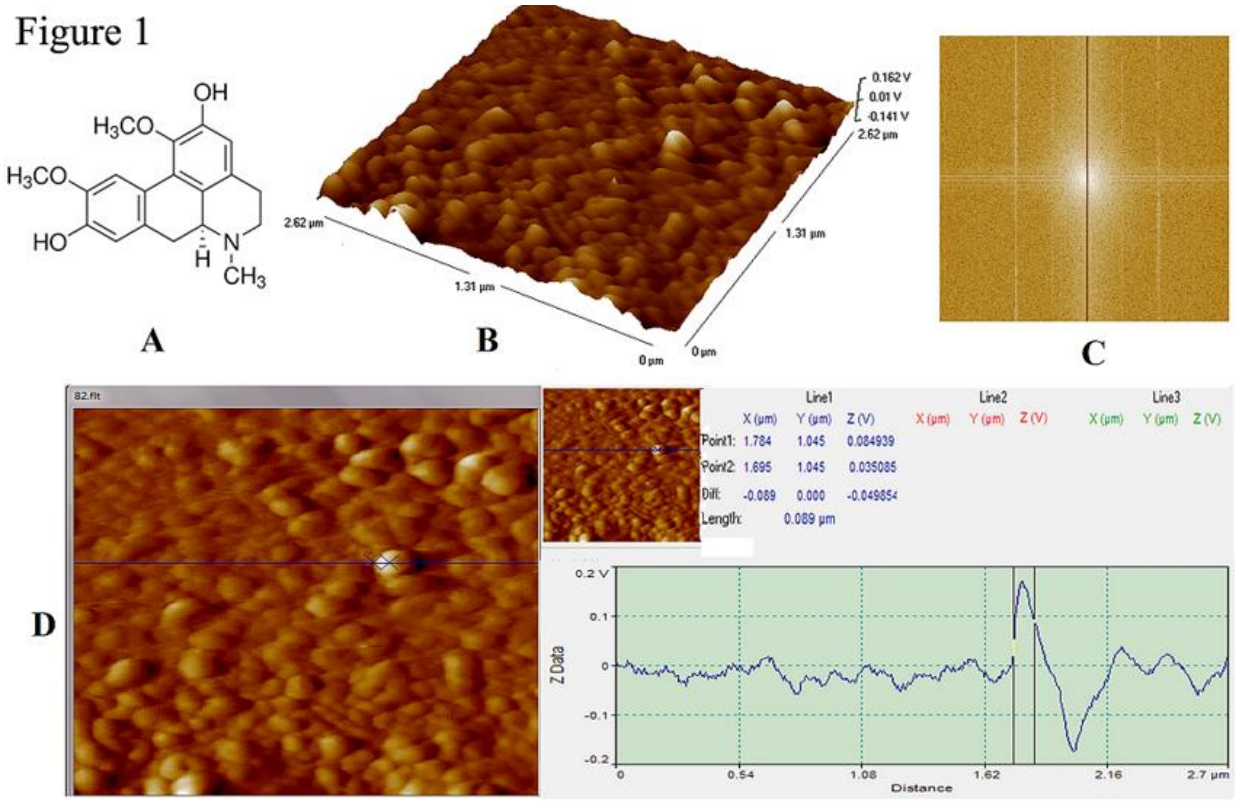

Figure 1. Chemical structure Boldine (A) and biophysical characterization of PLGA encapsulated Boldine nano- particles; (B) Atomic force microscopic image in three-dimensional mode. (C) Atomic force microscopic image in two-dimensional Fast Fourier Transformation (FFT) mode showing the frequency of occurrence of same-sized nanoparticles. (D)Atomic force microscopic image measured size of a single nano-particle by line measure method.

In vitro release of Boldine from nanoparticle system

The drug release from PLGA nanoparticles usually occurs in a biphasic manner, with an initial burst phase followed by a diffusion-controlled slower drug release phase. An initial burst phase was observed within 2 h due to the drug desorption and release from the nanoparticle surface. The sustained release of drug (Boldine) from PLGA polymer in the first 72 h was graphically illustrated in (Figure 3A).

Time of cellular entry

The times required for cellular uptake of Bol and NBol were determined by fluorescence microscopic study (Figures 3B-C). Results indicated that the rate of entry into the cells started increasing after 1 h onward for NBol and 3 h onward for Bol; cells became saturated after 6 h with both capsulated and unencapsulated forms of the drug. NBol treated cells showed increase of fluorescence intensity compared to Bol treated cells at different time intervals. Thus results indicated that NBol entered cells faster than did Bol.

Determination of cytotoxicity

Treatment with cisplatin resulted in a dose-dependent reduction of cell viability in both HepG2 and WRL-68 cell lines (Figure 4A) [2]. The LD50 values

calculated for cisplatin for HepG2 was (21.5±0.3) µmol/L and for WRL- 68 cells was (20.7±0.5) µmol/L. Treatment with Bol and NBol also resulted in a dosage-dependent reduction in cell viability for both HepG2 and WRL- 68 cells (Figure 4 B-C). LD_{50} values of Bol were calculated as 114.9±7.9 µg/mL and 130.8±10.1µg/mL for HepG2 and WRL-68 cells, respectively. With a view to detecting the optimum dose of the drugs showing maximum benefit of reduction of cisplatin induced cytotoxicity, we used a constant level of cisplatin for each trial, and varied the doses of Bol from 0 to 90µg/mL. We used 20µmol/L of cisplatin, the LD_{50} dose for WRL-68 cells. Co-treatment of Bol with cisplatin (20µmol/L) at moderate doses (54–72µg/mL) increased the viability of normal liver cells, but it did not affect cytotoxicity of cisplatin produced in the cancer cell line (Figure 4 D). However, at higher concentrations of Bol (81-90µg/mL), the viability of WRL-68 cells also got reduced, which led us to use the lower dose of Bol that gave the better result. These data suggest that a low dose of Bol (63µg/mL) along with the LD50 dose of cisplatin could be the most suitable combination to reduce the toxicity of cisplatin in normal cells while retaining its cytotoxic effect against cancer cells.

Figure 2

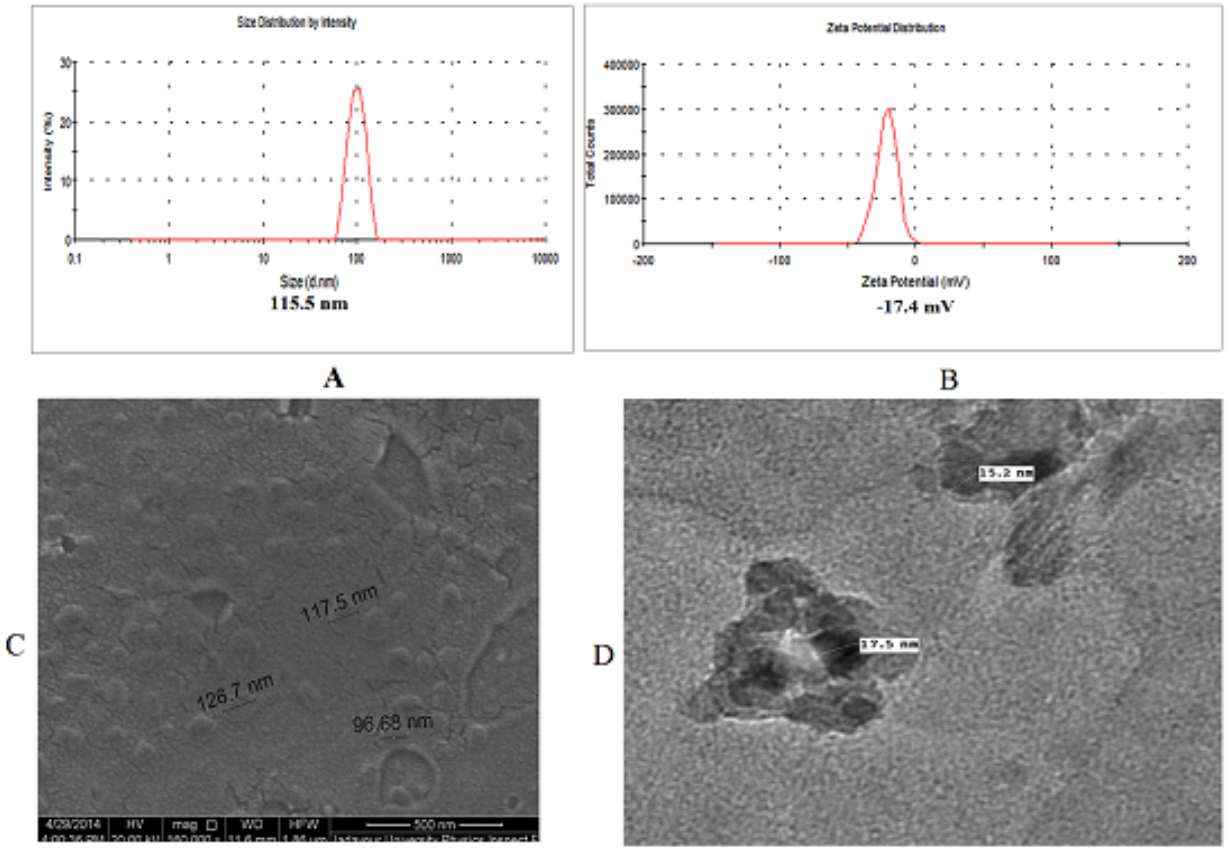

Figure 2. (A) Average particle size (Z-average-value) obtained from DLS data. (B) Graphical representation of zeta potential value. (C) Scanning electron microscopy of nano-particle. (D) Transmission electron microscopy of nano-particle

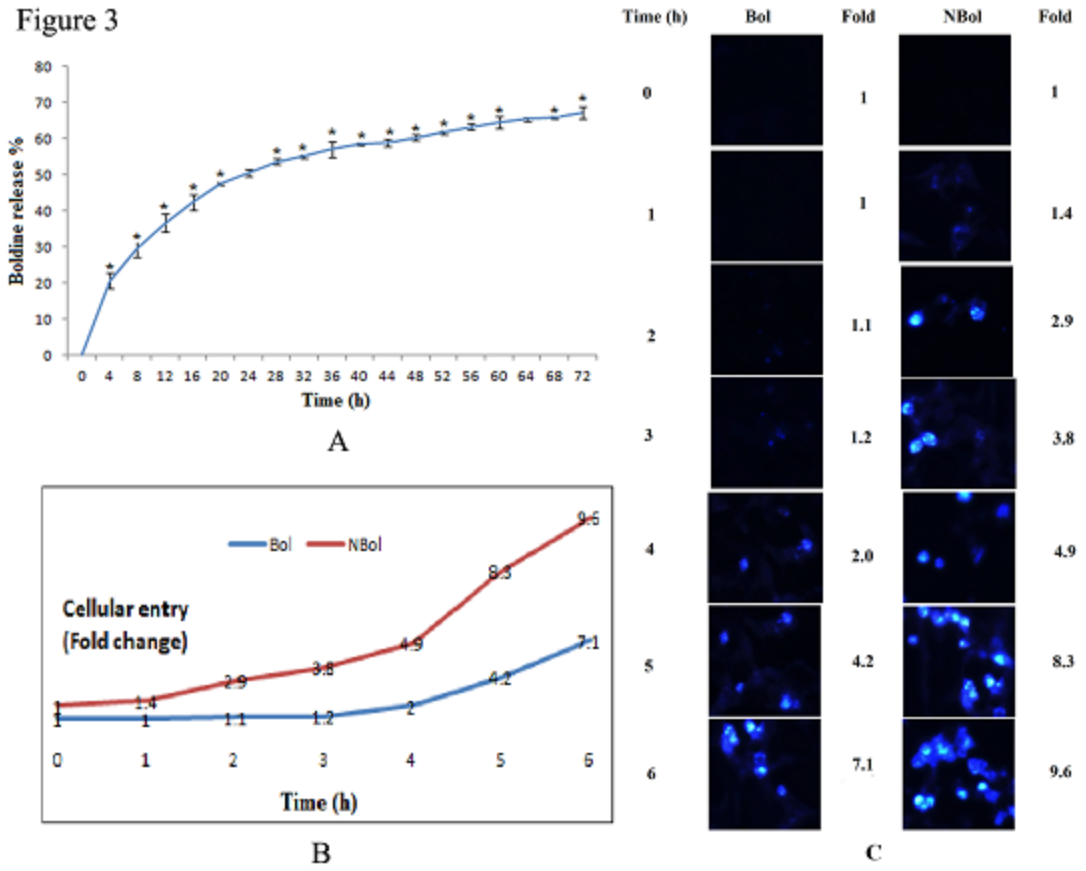

Figure 3. (A) *In vitro* release kinetics of Boldine from nano encapsulation for 72h time period. (B) Graphically represented fold change of cellular entry of Boldine (Bol) and nano-Boldine (NBol) at different time intervals. (C) Cellular uptake of (Bol) and (NBol). After incubation with Bol and NBol, WRL-68 cells were harvested at different time intervals and the cellular uptake was observed by fluorescence microscopy (blue fluorescence).

Similarly, the cytotoxicity of different doses of NBol (0-142µg/mL) was tested separately in the two cell lines (Figure 4 C). LD_{50} values of NBol were calculated as 118.3±8.9 µg/mL and 116.7±5.1µg/mL for WRL-68 and HepG2 cells, respectively. Results indicated that a higher dose of NBol treatment caused cytotoxicity in normal and cancer cells, killing > 50% of cell population. Therefore as NBol at a higher dose had cytotoxic effect of its own, though at a reduced degree as compared to cisplatin, and as our goal was to reduce overall cytotoxicity in the normal cells, we used a much lower dose of NBol (that gave us the best protective action) along with LD50 dose of cisplatin (Figure 4 E). Result of assay of conjoint treatment groups revealed that the optimum doses of NBol for increase of viability of normal cells were in the range from 18 to 36 µg/mL. Co-administration of NBol reduced cisplatin toxicity without affecting the cytotoxicity of cisplatin on the cancer cell lines (Figure 4 E). Cotreatment of NBol (18 to 36µg/mL) (compared with 0µg/mL) along with cisplatin increased the viability of normal liver cells up to 80.5%, but the viability of cancer cells remained almost same as that of only cisplatin treated lot.

The dose of NBol (27µg/mL) gave the best results. Therefore, this dose was picked up for the conjoint treatment with cisplatin, throughout the study. For a precise comparison of results, therefore, the same dose of 27µg/mL was selected for both Bol and NBol along with the uniform LD_{50} dose of cisplatin (20µmol/L) to examine relative efficacy of Bol and NBol for various parameters of study.

Figure 4

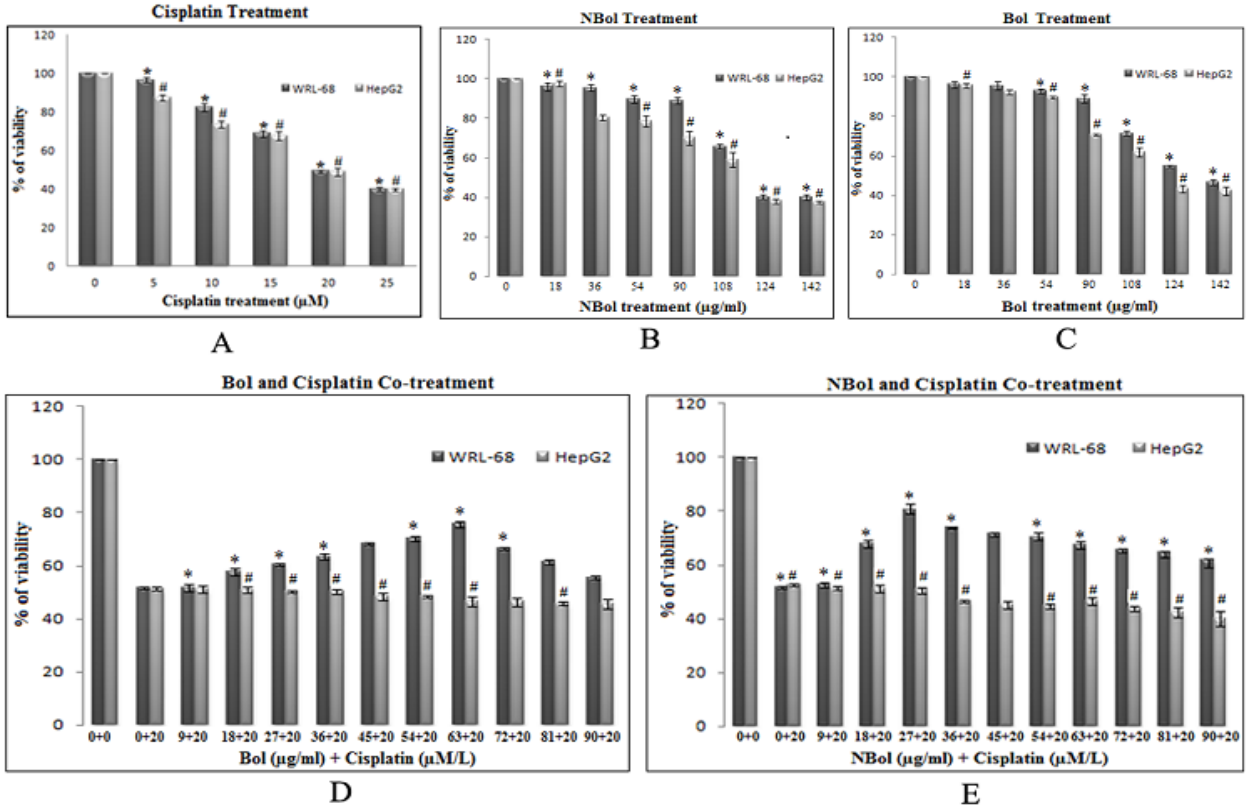

Figure 4. Cell viability assay by MTT; Cell viability was obtained by MTT with different doses of cisplatin (A), Boldine (Bol) (B), and NBol (NBol) (C) on normal (WRL-68) and cancer (HepG2) cell lines. Co-treatment of cisplatin with Bol (D) and cisplatin with NBol (E) on WRL-68 and HepG2 cells. Data are represented as percentage of control and are presented as mean±standard error of mean. *P<0.01 (for WRL-68) versus untreated control and #P<0.01 (for HepG2) versus untreated group was considered statistically significant.

Morphological changes

Morphological studies on normal and cancer cells were performed by phase-contrast microscopy. Data showed that there were some significant morphological changes among cisplatin, cisplatin plus Bol and cisplatin plus NBol groups for normal cell line. Treatment with cisplatin alone induced disruption of cellular membranes with bleb formation, and reduction of integrity of both cancer and normal cells. Protective effects on morphology were manifested in normal cell lines when cisplain was co-administered with Bol and NBol, more obvious in the latter group, but not much in cancer cell line (Figure 5A).

Inter cellular GSH depletion

When only cisplatin was administrated to WRL-68 and HepG2 cells, increased GSH depletion relative to the control treatment

was encountered. Co-treatment of NBol with cisplatin reduced rates of GSH depletion more strikingly than when compared against the cisplatin control, or Bol treated with cisplatin groups (Figure 5B).

In vitro expression of proteins related to cytotoxicity

Treatment of only cisplatin caused an increase in Bax expression; a decrease in cytochrome c within mitochondria was observed compared to its levels found in the cytosol as a result of cisplatin treatment. Contrary to this effect, co-administration of cisplatin with either Bol or NBol suppressed Bax expression and cytochrome c translocations in the normal WRL-68 cells. Further cisplatin-treatment up-regulated caspase 3 activity in normal liver cells, but co-administration of Bol with cisplatin or NBol with cisplatin tends to normalize caspase 3 activities (Figure 6A).

Cytochrome c release into cytosol

Confocal microscopical data confirmed an increase in Cytochrome c expression in the cytosolic fraction whereas decrease of expression in mitochondrial fraction could be observed in cisplatin alone treatment group, when compared with control. However, in the co-treatment group of cisplatin plus NBol, the elevated level of expression of cytochrome c was found to be down-regulated in the cytosolic fraction more significantly than in the treatment group of cisplatin plus Bol (Figure 6B).

Figure 5

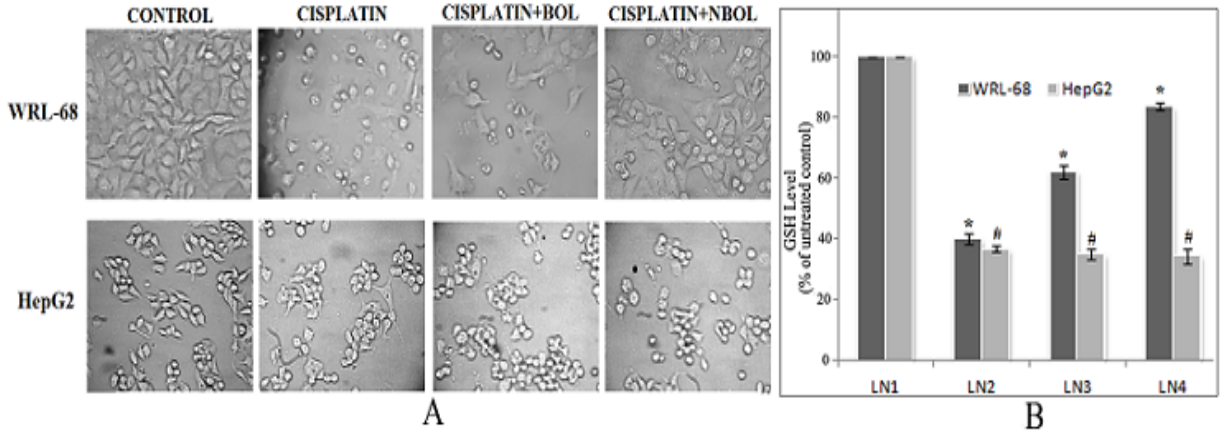

Figure 5. (A) Morphological analysis; the microscopic analysis of cispaltin (20µmol/L), cisplatin(20µmol/L) with Bol (27µg/mL) and cisplatin (20µmol/L) with NBol (27µg/mL) treated WRL-68 and HepG2 cells were observed by phase contrast microscope. Cisplatin induced damage to cellular structure of both WRL-68 and HepG2 cells. Co-administration of NBol prevented the damage of cellular structure induced by cisplatin in WRL-68 cells but not in HepG2 cells more remarkable than Bol. (B) GSH depletion in WRL-68 and HepG2 cells [LN1=control. LN2=cisplatin, LN3=cisplatin+Bol, LN4=cisplatin+NBol]. Data are represented as percentage of control and are presented as mean ± standard error of mean. Statistical significance was considered as *P<0.01 (for WRL-68) or #P<0.01 (for HepG2) versus untreated control.

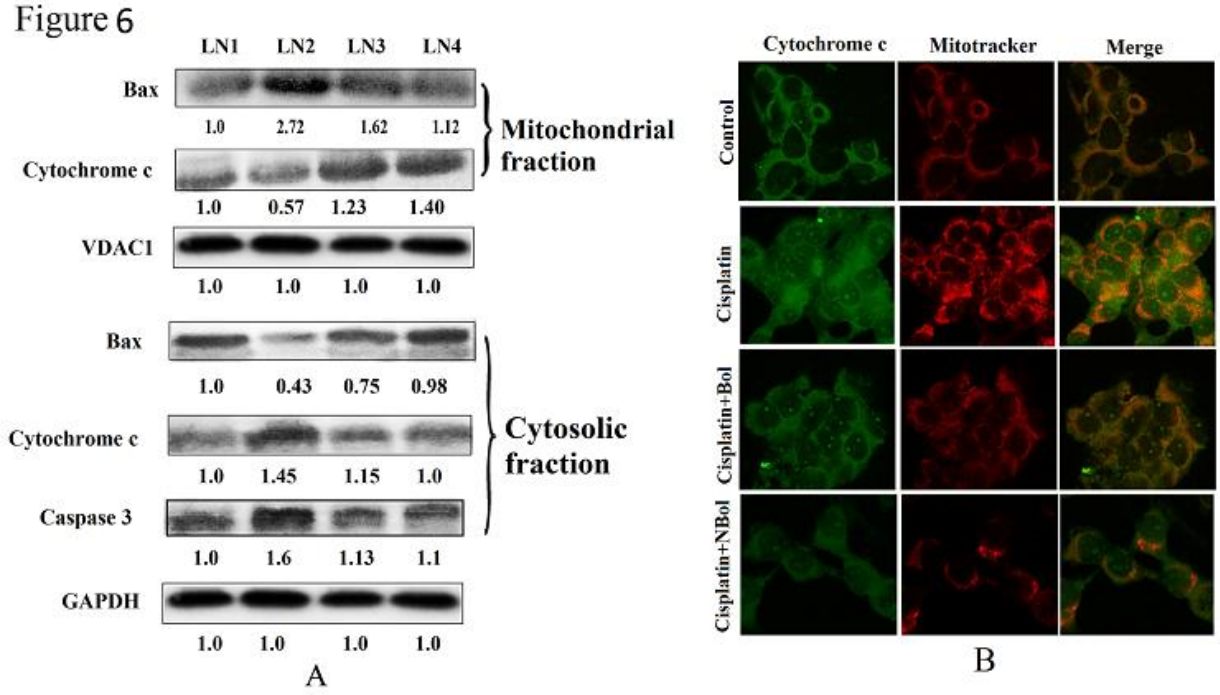

Figure 6. (A) Protein level expression study by western blot analysis: cytosolic and mitochondrial Bax and cytochrome c activities were analyzed, along with caspase 3 activity. VDAC1 (voltage-dependent anion-selective channel protein 1) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) served as loading control for mitochondrial and cytoplasmic fraction, respectively. The band intensity of the control was normalized to 1 and band intensity from treated cells was compared to the respective control [LN1=Control, LN2=Cisplatin (20µmol/L), LN3=Cisplatin (20µmol/L)+Bol (27µg/mL), LN4=Cisplatin (20µmol/L)+NBol (27µg/mL)]. (B) Confocal microscopic analysis; expression and localization of cytochrome c in mitochondria and cytosol in normal cells (WRL-68) were studied after treatment with cisplatin, co-administration of cisplatin with Bol and NBol, respectively. Release of cytochrome c was observed in cisplatin alone treated group where in co-administration of cisplatin with Bol and NBol groups, respectively, normalization of cytochrome c level was observed when compared to control set.

4. Discussion

Boldine had not been tested for its possible anti-cancer or cytoprotective potentials earlier. Nor was there any attempt to nano-encapsulate it with PLGA and characterize it physic-chemically. In this study, nano-encapsulation of Boldine was successfully done. The particle size was found to be in the range which is generally considered suitable for effective drug delivery. Further the negative zeta potential of the particles gives them an added edge for piercing the cell membrane, because of positive electrical charges of the cell membrane. Therefore, the time taken for cellular entry appeared to be quite less for making drug bioavailability. Interestingly, co-administration of a very low dose of Boldine (63µg/ml), appeared to have a reducing effect on cytotoxicity induced by cisplatin in normal liver cells, WRL-68

while it did not have any protective effect on the cancer cells (HepG2). This property of preferential protection appeared to be enhanced further when the PLGA-loaded nano Boldine was administered at a still lower dose (27µg/ml) along with cisplatin. Thus NBol appears to have greater potential as a supportive drug for the effective and longer use of the orthodox anti-cancer drug cisplatin, the use of which is often precluded or restricted for its severe side-effects and generation of hepatotoxicity.

This inference was further supported by results of viability assay, which clearly showed that only cisplatin treatment caused considerable reduction in viability of liver (HepG2) cancer cells, as well as of normal liver cells (WRL-68). But when Bol was co-administered with cisplatin at a very low dose (63µg/L) , the viability of WRL-68 was palpably increased (up to 75.6%) while the viability of cancer cells remained more or less the same as that of only cisplatin treatment series. Likewise, co-administration of NBol increased the survivability of WRL-68 cells to 80.5%, but did not significantly alter survivability of cancer cells.

Cytotoxicity and hepato-toxicity generation is related to generation of reactive oxygen species (ROS) and depletion of GSH level [12; 13]. Glutathione is an antioxidant enzyme often referred to as the body's "master antioxidant" due to its central role in protecting the body's cells from free radical damage. Glutathione is composed of the amino acids cysteine, glutamine and glycine and is concentrated in the liver, although it carries out its work throughout the body [14]. This important enzyme is involved in protecting cells from different toxic materials [15]. ROS generated in the cells resulted in the depletion of GSH level in liver tissue, which further helps in Bax translocation from mitochondria to cytosol to induce apoptosis. In this study, we found that ROS generation was increased along with the reduction in GSH level in the cisplatin-treated cells. ROS level was reduced and the GSH level increased when cells were subjected to combined treatment of Bol and cisplatin. This finding supports the ability of Bol and NBol to combat generation of ROS by cisplatin.

Both these events are also known to provoke Bax translocation from mitochondria to cytosol indicating the loss of mitochondrial membrane potential and release of cytochrome *c* to cytosol [16], resulting in an increase in cytosolic Bax and cytochrome *c* levels in cisplatin treated cells. Co-administration of NBol and Bol, hindered these processes and thus were able to protect the normal cells from cisplatin toxicity.

In recent years, technologial advancements have brought about many new innovative drug delivery systems; biodegradable and biocompatible polymers offer a suitable option for controlled targeted drug delivery. Nanocarriers appear to be a promising system for several advantages, such as increased protection of encapsulated drug targeting specific site for localized action [17]. In recent years, several approaches have been made by utilizing nanotechnology as a basic as well as applied science tool [18; 19] in therapeutic applications. However, among several known carriers, PLGA has gained importance for the encapsulation of a wide variety of drugs as it is biodegradable, biocompatible, capable of controlled release of the incorporated entity, and considered efficient carrier system for the delivery of drugs within the

cells [20] and less toxic [21] in nature. Further, PLGA has the ability to form stable nanoparticles, and has already been approved for use in humans by the US Food and Drug Administration [22].

PLGA-loaded nanoparticles were characterized to preview possibility of their use as an effective nanocarrier system. The particle size is an important parameter as it can directly affect physical stability, cellular uptake, and drug release from the nanoparticles and can affect its bio-distribution [23]. DLS results suggested the particle size of NBol to be small with rounded smooth surface, and with negative zeta potential, ideally suited for speedy cellular entry. The dispersion pattern in NBol also exhibited a unimodal particle size distribution, as confirmed by the low polydispersity index (PDI: 0.217 ± 0.016). Throughout this study, the effects of NBol were compared with those of Bol in respect of drug delivery and hepatoprotective capabilities. Incidentally, fate of the drug loaded polymeric nanoparticles primarily depends on its physicochemical character. Present findings suggested that stable nanoparticles of NBol were successfully formulated. A carrier that could slowly release the drug at the site of action in the intracellular compartment would enhance the curative efficacy of the drug as well as could sustain its prolonged therapeutic effects [24; 25]. PLGA is known to undergo bulk degradation and release of the entrapped drug molecules from PLGA matrix has been found to occur through diffusion-cum-degradation mediated process [26; 27]. Our results demonstrated that NBol have a much lower protective dose as compared to that of Bol in combination with cisplatin. Further, images of intracellular uptake of Bol and NBol in HepG2 cells through fluorescence microscopic analysis confirmed that cellular uptake of NBol was more rapid and pronounced when compared to that of Bol. This phenomenon suggests that NBol could amplify cellular uptake of the drug, resulting in increased preferential cytoprotective action in normal cell. Further, the dose that gave optimum results was also very low. Therefore, overall results point out that both Bol and NBol could act as a supportive drug in cisplatin oncotherapy, but NBol had much greater efficacy at a lower dose.

Further animal experiments will be required before these drugs could finally be recommended for possible human trial and use.

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Conflict of interest

There is no conflict of interest to declare.

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